Studies of Cicatricial Pemphigoid Autoantibodies Using Direct Immunoelectron Microscopy and Immunoblot Analysis

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We studied 11 consecutive patients with classical cicatricial pemphigoid (CP) using direct immunoelectron microscopy (IEM) and Western immunoblotting analysis. Direct IEM performed in the skin or gingival mucosa revealed in all 11 CP patients that immunoglobulins and complement deposits were usually thick and discontinuous along the dermoepidermal junction, mostly localized on the lamina densa and occasionally in the lamina lucida. By direct IEM, the ultrastructural aspect in CP differs from the pattern observed in bullous pemphigoid (BP) and from that of chronic epidermolysis bullosa acquisita (EBA). Nine CP patients were studied by Western immunoblotting and, of these nine, only two had detectable anti-basement membrane zone (BMZ) antibodies by indirect immunofluorescence on salt-split skin. By immunoblotting performed on protein extracts of heat-separated epidermis, eight out of the nine CP sera specifically reacted with two protein bands of approximately 230–240 kD and 180 kD, similar to those recognized by BP sera in co-migration experiments. By immunoblotting on skin BMZ extracts, none of these nine CP sera recognized the 290-kD major polypeptide of EBA antigen. Taken together, these results suggest that, in CP, the target-antigen, as identified on immunoblots, is similar to BP antigen, but with an abnormal expression within the dermoepidermal junction of patients, which may in part explain the scarring course of the disease. *J Invest Dermatol 94:630–635, 1990*

Cicatricial pemphigoid (CP) is an uncommon variant of autoimmune subepidermal bullous disease characterized by scar formation, primarily involving oral and ocular mucous membranes and, less frequently, the skin [1,2]. As in bullous pemphigoid (BP) and epidermolysis bullosa acquisita (EBA), patients with CP have in vivo-bound immunoglobulins and/or complement along the basement membrane zone (BMZ) of affected tissues. Although the disease is probably the result of an autoimmune process, sera from CP patients show very inconstantly circulating anti-BMZ autoantibodies by indirect immunofluorescence (IIF) on normal human skin [3]. In addition, only a little information is available concerning the target-antigen(s) of the dermo-epidermal junction (DEJ) which are involved in CP. On one hand, previous direct immunoelectron microscopy (IEM) studies of patients with CP reveal localization of the immunoreactants within lamina lucida of the DEJ [4,5], a pattern similar to that reported in cases of BP [6,7]. But, direct immunofluorescence studies on mechanically induced suction blisters performed by Fine et al suggested that, despite a common localization within the lamina lucida of the DEJ, the antigen involved in CP may be distinct from the BP antigen [5]. The same authors confirmed these findings using indirect immunofluorescence on salt-split skin, and even suggested the presence of at least two CP antigens [8]. On the other hand, preliminary westernblot analysis on protein bands from normal human epidermis [9,10] suggested that both CP and BP autoantibodies could recognize a 240-kD band, which represents the major determinant of BP antigen [11]. In the present work, we analyzed both ultrastructural and biochemical features of involved target-antigens in 11 patients with CP. For this study, we performed direct IEM to determine the ultrastructural in vivo localization of immunoreactants. In addition, sera of these patients were studied by western immunoblotting on protein extracts from both heat-separated normal epidermis and skin BMZ to partially characterize the target antigen(s) recognized by CP autoantibodies.

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Abbreviations:
BMZ: basement membrane zone
BP: bullous pemphigoid
CP: cicatricial pemphigoid
DAB: diaminobenzidine
DEJ: dermo-epidermal junction
EBA: epidermolysis bullosa acquisita
IEM: immunoelectron microscopy
IIF: indirect immunofluorescence
PAGE: polyacrylamide gel electrophoresis
SDS: sodium dodecyl sulfate

MATERIALS AND METHODS

Patients Eleven consecutive patients (seven female, four male) with a mean age of 70.3 years (range, 26–88) who met the clinical and immunopathologic diagnosis criteria of CP were studied.

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Among the patients with subepidermal bullous disease demonstrating in vivo immunoglobulins and/or complement deposits along the BMZ of epidermis, we selected those with classical CP on the basis of 1) the predilection for mucosal involvement (especially oral and ocular) contrasting with a less important skin involvement, and 2) the chronic and scarring course. Clinical and immunopathologic data are summarized in Table I. Ocular lesions included conjunctival blisters, synechia, or symblepharon. Oral involvement was characterized by erosions, blisters within the oral cavity, and/or desquamative gingivitis. Genital lesions included chronic erosions and/or blisters of the external genitalia. Skin involvement always predominated on the face, head, and trunk, and consisted of rare blisters, leading to persistent erosions which healed with atrophic scars. Immunofluorescence data are summarized in Table I. By direct immunofluorescence, six out of 11 CP patients also had linear IgA deposits along the BMZ in addition to IgG and C3 (Table I).

Sera Sera were obtained from nine out of the 11 CP patients, at time of diagnosis, and stored at −70°C until used for IIF or western immunoblotting studies. Sera from patients with typical clinical, immunologic, and immunoelectron microscopic features of BP (n = 10) and EBA (n = 3), and from healthy subjects (n = 20) served as controls. By standard IIF [12] on normal human skin, none of the patients had circulating anti-BMZ antibodies. Using IIF on 1.0 M NaCl separated skin [13], two CP patients had circulating anti-BMZ antibodies, of the IgG class, at a low titer (1:40), with a mixed (both epidermal and dermal side) staining pattern (patient number 4) or an epidermal side staining (patient number 11). EBA sera which contained anti-BMZ antibodies (n = 3) bound to the base of the split skin, at titers of 1:10 to 1:40, whereas BP sera bound to the roof.

Immunoelectron Microscopy Biopsies were obtained from peribullous skin, and/or from gingigal mucosa (patients numbers 2 and 7) and processed according to a previously described direct IEM technique [14]. In brief, five 0.7-mm-thick slices of skin were obtained from each 6-mm punch biopsy with a specially designed hand microtome [15]. The slices were incubated with peroxydase-conjugated anti-human γ and α goat polyclonal antibodies (Institut Pasteur, Marnes la Coquette, France), and anti-human C3 rabbit polyclonal antibody (Dako Laboratories) at a dilution of 1:10. As controls, two slices from each patient's biopsy were respectively incubated with anti-human anti-μ goat polyclonal antibody (Institut Pasteur, Marnes la Coquette, France) and with Hank's medium. Direct IEM study of skin samples from healthy subjects, and from patients with typical bullous pemphigoid (BP) or epidermolysis bullosa acquisita (EBA) served as additional controls. All the ultrathin sections were examined without staining, with a Philips EM 301 electron microscope.

Western Immunoblotting

Preparation of Epidermal Extracts Surgical specimens from dermolipectomy of normal human skin were used as sources of skin. Dermo-epidermal separation was obtained by heating [16]. Epidermal proteins were extracted in 65 mM Tris-HCl (pH 6.8) buffer containing 2% SDS, 10 mM ethylenediamine tetraacetate, 2 mM phenylmethyl sulfonyl fluoride and 0.01 mg/ml of the following protease inhibitors: pepstatin, antipain, chymostatin, and leupeptin (Sigma Chemical Co.) The homogenate was ultrasonicated (4 × 15 sec), then centrifuged at 10,000 g for 20 min.

Preparation of Skin BMZ Extracts Skin BMZ extracts, which contain the EBA antigen as defined by Woodley et al [17], were prepared from surgical specimens of normal human skin, according to the method of Stanley et al [18]. In brief, skin was incubated in 1.0 M NaCl containing 1 mM phenylmethyl sulfonyl fluoride and 2 mM ethylenediamine tetraacetate, at 4° for 72 h, after which the epidermis was removed. Then, the skin BMZ was extracted for 1 h at room temperature with a 0.065 M Tris-HCl pH 6.8 buffer containing 2% SDS, 8 M urea, 0.1 M dithiotreitol, 1 mM phenylmethyl sulfonyl fluoride, and 2 mM ethylenediamine tetraacetate.

SDS-PAGE and Western Immunoblotting Epidermal and skin BMZ extracts were subjected to a SDS-PAGE as described by Laemmli [19] after reduction with 0.1 M dithiotreitol, using slab gels with 5% polyacrylamide. Then, epidermal and skin BMZ protein extracts were electrophoretically transferred onto nitrocellulose paper as described by Towbin [20], and stained by the peroxydase method using a peroxydase-conjugated anti-human IgG (H + L) goat antibody (Institut Pasteur, Marnes la Coquette, France), with CP and control sera as previously described [21]. Sera were tested at least twice at the dilutions of 1:50 and 1:100.

RESULTS

Direct Immunoelectron Microscopy Table II summarizes the results of direct IEM studies in CP patients. In each patient concordant results were obtained from skin or buccal mucosa (patient number 2), and from different biopsy samples incubated with anti-γ, -α, or C_3. Diaminobenzidine (DAB) deposits were always localized on the lamina densa, which consequently disappeared under them (Fig 1). DAB deposits were seen both on the lamina densa and in lamina lucida in eight out of the 11 patients (Figs 1 and 2). They were only located on the lamina densa in one case (patient 10). Finally, in two patients (numbers 1 and 2), deposits overflowed under the lamina densa in the anchoring fibril zone (Fig 3). When a dermoepidermal separation occurred, it was in lamina lucida, and deposits were distributed on both the dermal and the epidermal side (Fig 4). A striking feature was that DAB deposits were usually very thick (Fig 1), but irregular in thickness and discontinuous along the

Table I. Cicatricial Pemphigoid: Clinical and Immunofluorescence Data

Patient Number/Sex/Age	Skin Bullae	Mucosal Involvement	Scar	Milia	Direct IF			Indirect IF
					IgG	IgA	C3	
1/M/62	rare	oral, ocular	+	+	+	+	+	−
2/M/76	rare	oral, ocular	+	−	+	+	+	−
3/M/76	numerous	oral, ocular	+	+	+	+	+	−
4/M/84	rare	oral, genital	+	−	+	+	+	1:40[a]
5/F/72	rare	oral	+	+	+	−	+	nd[b]
6/F/75	rare	oral, ocular	+	−	+	+	+	−
7/F/26	absent	oral[c]	+	−	−	−	+	nd
8/F/80	rare	oral	+	+	+	−	+	−
9/F/88	rare	oral, genital	+	−	+	+	+	−
10/F/59	rare	oral, genital	+	−	+	−	+	−
11/F/78	rare	oral	+	+	−	−	+	1:40[a]

[a]Positive only when performed on 1.0 M salt-split skin as described by Gammon [12].
[b]Not done.
[c]Desquamative gingivitis.

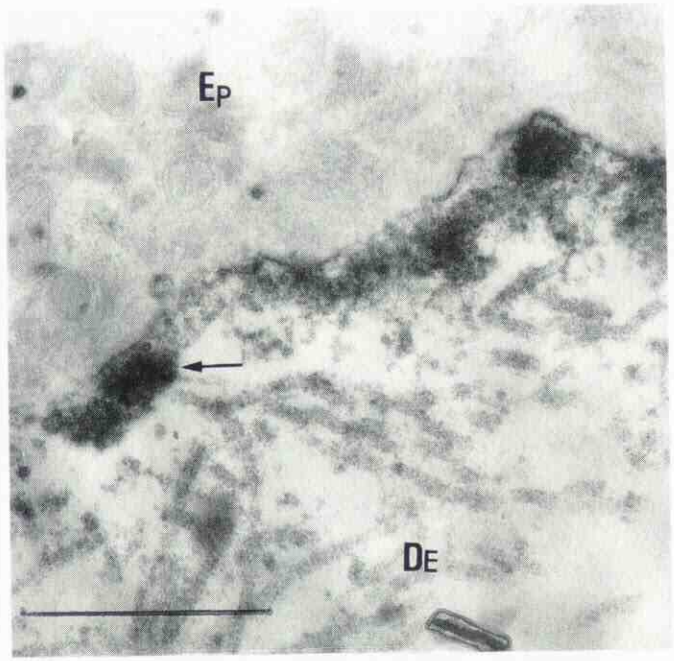
Table II. Direct Immunoelectron Microscopy Data of Patients with Cicatricial Pemphigoid

Patient Number	Localization of the Immunoreactants		
	LL[a]	LD[b]	AFz[c]
1	−	+	+
2	+	+++	+
3	+	++	−
4	+	+	−
5	+	+	−
6	+	+	−
7	+	+	−
8	+	+	−
9	+	+	−
10	−	+	−
11	+	+	−

[a]Lamina lucida.
[b]Lamina densa.
[c]Anchoring fibril zone.

DEJ (Figs 2 and 3). This ultrastructural aspect of in vivo bound immunoreactants in CP differs from the pattern observed in BP, in which deposits are less thick, generally more continuous, in the lamina lucida above a well-visible lamina densa (Fig 5). The direct IEM aspect of CP patients also differs from that of chronic EBA, in which DAB deposits are very thick and localized mostly below the lamina densa. No DAB deposit was found in biopsy samples from normal subjects.

Western Immunoblotting Table III summarizes the results of the western immunoblotting experiments. By immunoblotting on protein bands of heat-separated normal epidermis, circulating antibodies were demonstrated in sera of eight of the nine tested CP patients. Six CP sera specifically labeled a band of approximately 240 kD, which usually appeared similar to that recognized by most BP sera in co-migration experiments (Fig 6), and represents the major determinant of BP antigen [11]. Depending on experiments, the staining pattern of this 240-kD band by some CP sera (from patients 2 and 9) was not strictly identical to that obtained with BP sera (as shown in Fig 7). Three CP sera labeled an additional broad band of approximately 180 kD, similar to a minor determinant recognized by some BP sera in co-migration experiments (Fig 6). As previously discussed [21], our calculation of the Mr of this 180-kD band was generally slightly lower (in a 165–180 kD range) than reported by Labib et al [9]. But since the Mr are roughly estimated anyway, we have chosen to label the Mr the same as Labib et al to prevent confusion. In addition, two CP sera reacted only with this 180-kD band. By western immunoblotting, reactivity of CP sera was often faint, when compared to that of BP sera (Fig 6). However, three CP sera demonstrated a strong positivity on immunoblots (as indicated by ++ in Table III), which, in some experiments, were at least greater than the reactivity of the control BP sera (*lane 2* in Fig 7*A*). Since none of normal sera recognized these bands, the 240- and 180-kD bands appeared specific for both BP and CP sera. Finally, by immunoblotting performed on BMZ extracts of normal human



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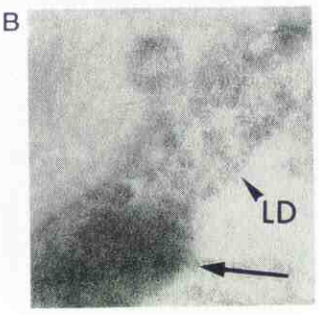
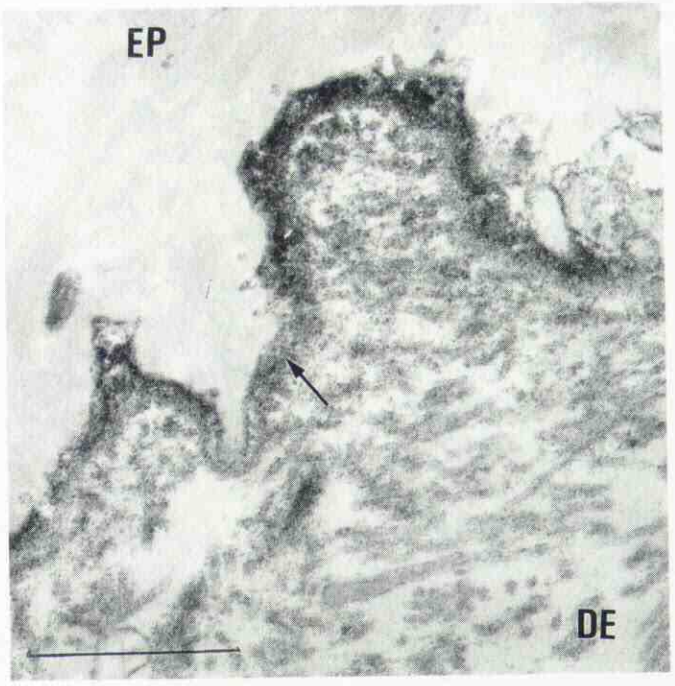


B

Figure 1. Direct immunoelectron microscopy (CP patient number 7): thick immunodense deposits *(arrow)* are mostly located on the lamina densa (LD), and partly obscure the lamina lucida. Note that lamina densa is markedly thickened in areas without deposits *(B)*. *A:* magnification × 33,600; *bar,* 1 μ. *B:* magnification × 90,000; *bar,* 0.25 μ.



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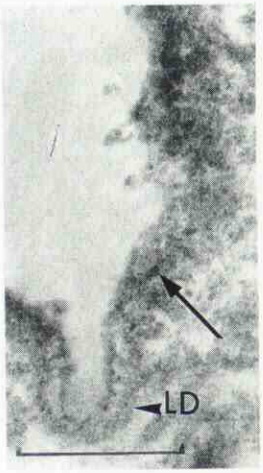

B

Figure 2. Direct immunoelectron microscopy (CP patient number 11): DAB deposits *(arrow)* are distributed both on the lamina densa (LD) and in the lamina lucida. Deposits are irregular in thickness and even discontinuous along the dermo-epidermal junction. *A:* magnification × 28,000; *bar,* 1 μ. *B: magnification* × 90,000; *bar,* 0.25 μ.

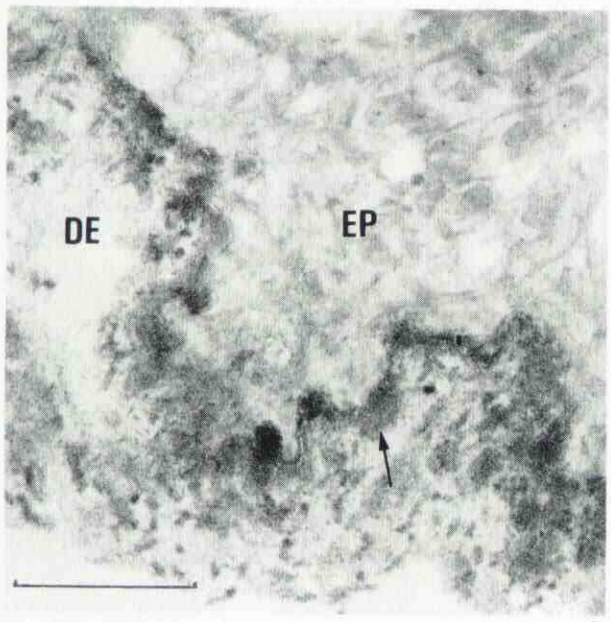

Figure 3. Direct immunoelectron microscopy (CP patient number 2): immunodense deposits are on the lamina densa (LD), and, below it, overflow in the anchoring fibril zone *(arrow)*. *A:* magnification × 27,000; *bar,* 1 μ. *B:* magnification × 60,000; bar, 0.25 μ.

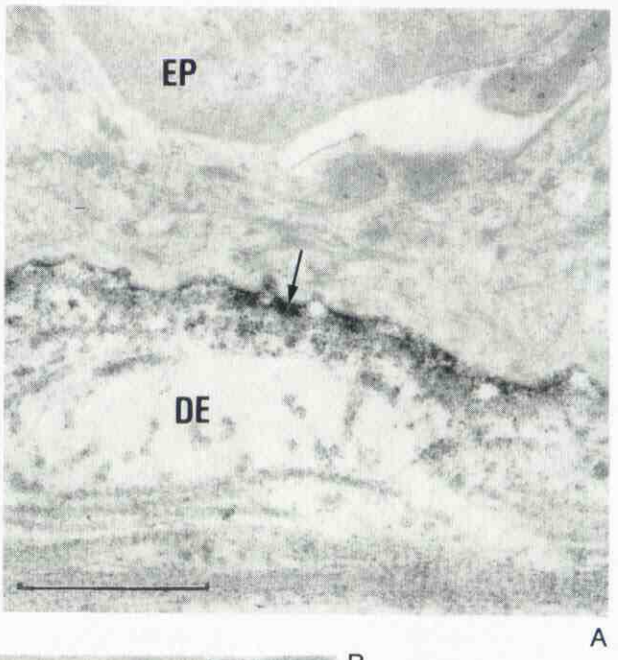

Figure 5. Direct immunoelectron microscopy of a BP patient: DAB deposits *(arrow)* are located in the lamina lucida. The lamina densa (LD) is well identified below immune deposits. *A:* magnification × 28,000; *bar,* 1 μ. *B:* magnification × 60,000; *bar,* 0.25 μ.

skin, no CP sera bound the 290-kD polypeptide of EBA antigen, whereas EBA sera did stain this band (Fig 7).

DISCUSSION

CP is a relatively rare form of autoimmune subepidermal bullous disease with in vivo bound immunoglobulins and complement along the BMZ of the epidermis and/or the mucosae. As opposed to BP, patients with CP have a particular predilection for mucosal involvement and a scarring course, and a low prevalence of detectable anti-BMZ antibodies by IIF using both normal human skin and oral mucosa as substrates [1,5]. This last point represents a handicap for the characterization of the target antigen involved in CP. Consequently, a direct IEM procedure is most suitable to investigate, in a non-selected group of CP patients, the in vivo localization of the involved target antigen. In this study, we used a direct IEM technique, whose results in a large series of patients with subepidermal autoimmune bullous disease have been recently reported in detail [15]. This technique permits a satisfactory preservation of the ultrastructural morphology, without freezing. Our results show that, in vivo, CP autoantibodies are on a target antigen which appears localized not only in the lamina lucida, but mostly on the

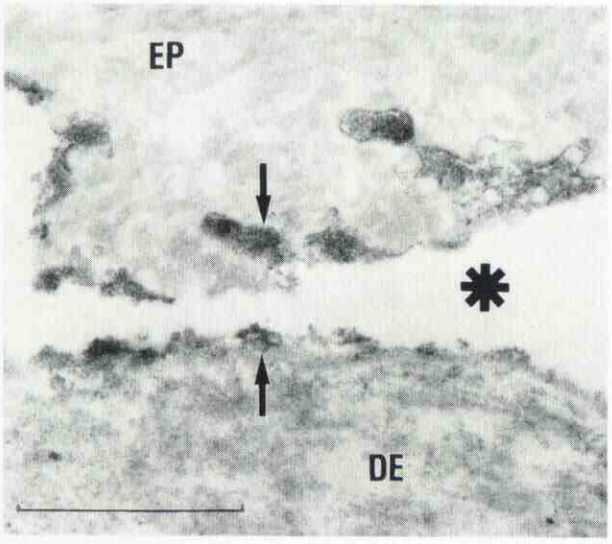

Figure 4. Direct immunoelectron microscopy (CP patient number 9): area of spontaneous dermo-epidermal cleavage in lamina lucida *(asterisk)*. Immunodense deposits are on both epidermal and dermal sides *(arrow)*. Magnification × 33,000; *bar,* 1 μ.

Table III. Western Immunoblotting Data of Patients with Cicatricial Pemphigoid

	Protein bands (kD) Identified by Immunoblotting[a]			
	Epidermal Extracts		Skin BMZ Extracts	
Patient Number	240	180	290	145
1	+	−	−	−
2	+	+	−	−
3	+	+	−	−
4	−	++	−	−
6	++	−	−	−
8	−	−	−	−
9	++	−	−	−
10	+	+	−	−
11	−	+	−	−

[a]Scored by visual comparison, from at least two determinations.

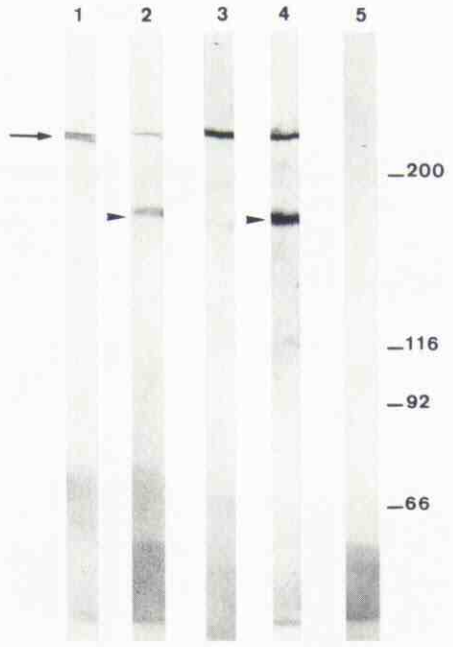

Figure 6. Immunoblot on identical lanes of heat-separated epidermal extract stained with CP sera (from patient number 1 on *lane 1*, and patient number 10 on *lane 2*), BP sera *(lanes 3 and 4)*, and normal human serum *(lane 5)*. Concurrently electrophoresed standards are indicated at right (kD). Two protein bands are specifically recognized by both CP and BP sera in this co-migration experiment: the 240-kD major polypeptide of BP antigen *(arrow)* and the 180-kD minor epitope of BP antigen *(arrowhead)*. Here, the staining of these bands by CP sera is significantly fainter than obtained with BP sera.

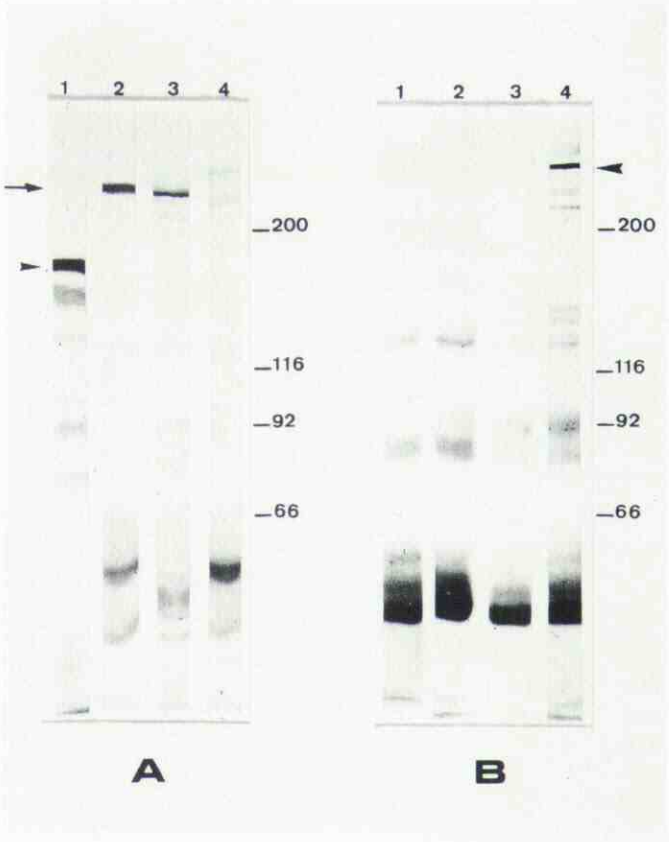

Figure 7. Comparison of heat-separated epidermal extract *(A)* and skin-BMZ extract *(B)* stained with identical sera. Concurrently run standards are indicated at right (kD). The following sera were tested: CP sera on *lanes 1 and 2* (from patients numbers 4 and 9, respectively), BP serum on *lane 3*, and EBA serum on *lane 4*. In *A*, the *arrow* indicates the 240-kD major polypeptide of BP antigen and the *small arrowhead* indicates the 180-kD minor epitope of BP antigen. In that experiment, the staining pattern of the 230–240 kD band obtained with the tested CP *(lane 2)* and BP *(lane 3)* sera is not fully identical. In *B*, the 290-kD major polypeptide of the EBA antigen *(large arrowhead)* is only stained by the EBA serum *(lane 4)*, but not by CP or BP sera.

lamina densa of the DEJ. This ultrastructural localization of the immunoreactants and their granular appearance have been previously noted by Albini et al in CP, who also observed, by place, the destruction of the lamina densa [22].

Similarly, in the Brunstig-Perry variant of CP, Murata et al have found that immunoreactants were localized in lamina lucida and occasionally extended on lamina densa and beyond, to the uppermost part of the dermis [23]. In addition, previous immunofluorescence studies of Fine et al had shown that CP autoantibodies could stain the base of a salt split skin, suggesting a deep localization of the CP antigen within the lamina lucida and/or on lamina densa [8]. In addition, the lamina densa is often abnormal, either duplicated, or partly or totally destroyed as previously reported [24,25]. This involvement of the lamina densa may explain the susceptibility to scar formation in CP. In our experience, the ultrastructural distribution of the immunoreactants in CP perceptibly differs from that observed by direct IEM in BP [6,7,15,22], in which they are exclusively localized within the lamina lucida. These differences by direct IEM had not been clearly reported by other investigators for whom DAB deposits were exclusively located within the lamina lucida, above the lamina densa, as in BP [4,5,26]. This discrepancy with our IEM data might be due to technical differences, in particular the freezing of skin biopsies before the incubation with the immunoreactants in previous studies [5,26]. On the contrary, our IEM data of CP patients are quite similar to those reported by Briggaman et al in the inflammatory stage of EBA [27]. But patients at this initial stage of EBA have generalized or acral distribution of blisters, without scarring, a clinical presentation quite distinct from that of our CP patients at the time of IEM study. In addition, unlike CP, sera of patients at this inflammatory stage of EBA contained circulating anti-BMZ antibodies demonstrable by IIF in most cases and recognized the 290-kD and 145-kD protein bands of EBA antigen by western immunoblotting [27].

So, the direct IEM pattern described in our study is not entirely specific for CP, that is, making essential the westernblot analysis of sera against both epidermal and skin BMZ extracts. Prior to this study, there have been two studies in which western immunoblotting data of CP patients have been reported [9,10]. In both of these studies, sera were only tested against epidermal extracts of heat-separated normal human epidermis. Four of the 11 tested CP sera reacted with a 240-kD band, which represents the major determinant of BP antigen. An additional 120-kD band was recognized by two CP sera [10], but six out of the 11 tested CP sera did not react with any bands. In our study, six out of nine CP sera recognized a band in the 230–240-kD range, as did most sera from patients with classical BP. But, some CP sera (from patients 2 and 9) showed by immunoblotting differences of the staining pattern of this 240-kD band when compared with BP sera (as shown in Fig 7). This could be due to minor biochemical differences of the target-antigen involved in CP and BP. But it could also signify binding of autoantibodies to an epitope unrelated to BP antigen, in some cases of CP. Further investigations (using immunoprecipitation procedures) are in progress to clarify this point. We also found that CP sera could react with a broad band of approximately 180 kD, which represents a minor epitope of BP antigen, as detected by immunoblotting [9,10]. None of our nine CP sera recognized the previously reported 120 kD [10]. In our CP patients, there was no obvious relation between the detection of the 180- or 240-kD bands on immunoblots and the deepness of the ultrastructural localization of immunoreactants by direct IEM. We also tested the CP sera by western immunoblotting on skin BMZ extracts of normal human skin to

determine whether these sera did recognize the EBA antigen or not. In these experiments, none of the CP sera reacted with the 290-kD or the 145-kD bands which defined the EBA antigen. Taking together these results, we conclude that antigen(s) involved in CP are at least partially identical to BP antigen and are distinct from EBA antigen. Finally, since CP sera generally did not reveal circulating anti-BMZ antibodies by IIF, their immunoblot analysis does confirm that the westernblot technique is more sensitive than IIF for the detection of autoantibodies against epidermal determinants [10,21,28].

Our westernblot data show that target antigens in CP are only present in protein extracts of normal human epidermis. Since proteins are extracted from epidermis after separation by heating, in which cleavage occurs in lamina lucida [16], one might conclude that target antigen(s) of CP is localized in the uppermost part of the lamina lucida of the DEJ in normal human skin and is identical to BP antigen, which is expressed in the hemidesmosomes of basal keratinocytes as well as within lamina lucida of normal human skin [6,7,15,29]. Unfortunately, our direct IEM data show that, in CP patients, immunoreactants are localized on lamina densa as well as in lamina lucida. Such a discrepancy between the in vivo localization (i.e., in the patients) of the target antigen as defined by direct IEM and its localization in normal skin has been recently reported by Horiguchi et al using both direct and indirect IEM analysis of BP patients [30]. As a conclusion, our study suggests that the target antigen involved in CP might be the BP antigen, but with an abnormal expression within the DEJ of patients with CP. Although further investigations are necessary to verify that hypothesis, it may explain, at least in part, the special scarring course of this disease.

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